Leaf micromorphology and anatomy of *Myrceugenia rufa* (Myrtaceae). An endemic coastal shrub of north-central Chile

Micromorfología y anatomía foliar de *Myrceugenia rufa* (Myrtaceae). Un arbusto costero endémico de la zona centro-norte de Chile

ABSTRACT

Species of fleshy-fruited Myrtaceae are generally associated with humid environments and their vegetative anatomy is mainly mesophytic. *Myceugenia rufa* is an endemic and rare species from arid zones of the coast of central Chile and there are no anatomical studies regarding its leaf anatomy and environmental adaptations. Here we describe the leaf micromorphology and anatomy of the species using standard protocols for light and scanning electron microscopy. The leaf anatomy of *M. rufa* matches that of other Myrtaceae, such as presence of druses, schizogenous secretory ducts and internal phloem. Leaves of *M. rufa* exhibit a double epidermis, thick cuticle, abundant unicellular hairs, large substomatal chambers covered by trichomes and a dense palisade parenchyma. Leaf characters of *M. rufa* confirm an anatomical adaptation to xerophytic environments.

KEYWORDS: hairs, leaf anatomy, *Myrceugenia rufa*, Myrtaceae, SEM, xerophytic characters

RESUMEN

Las especies con frutos carnosos de Myrtaceae son generalmente asociadas a ambientes húmedos y su anatomía vegetativa principalmente mesofítica. *Myceugenia rufa* es una especie rara y endémica de lugares secos de la costa de Chile central y no hay estudios anatómicos de sus hojas en términos de adaptaciones ambientales. En este artículo describimos la micromorfología y anatomía foliar de la especie usando protocolos estándar para microscopia óptica y electrónica de barrido. La anatomía foliar de *M. rufa* es similar a otras especies de Myrtaceae, en lo que respecta a presencia de drusas, canales secretores esquizógenos y floema interno. Las hojas de *M. rufa* tienen una epidermis doble, cutícula gruesa, pelos unicelulares abundantes, cavidades subestomáticas amplias y cubiertas por tricomas, y un denso parénquima en empalizada. Los caracteres foliares de la especie confirman una afinidad morfoanatómica a ambientes xerofíticos.

PALABRAS CLAVE: anatomía, caracteres xerofíticos, *Myrceugenia rufa*, Myrtaceae, SEM, tricomas,

INTRODUCTION

Myrtaceae Juss. is a pantropical family of trees and shrubs with approximately 5500 species, divided into two subfamilies, 17 tribes and 142 genera (Wilson *et al.* 2005; Govaerts *et al.* 2008). This family is particularly diverse in the southern hemisphere (Ladiges *et al.* 2003), with a high diversity in Central America (McVaugh 1968), South America (Landrum 1988) and Australia (Lucas *et al.* 2007; Thornhill and Crisp 2012). The members of this family are woody, with entire, simple, evergreen and mostly opposite leaves, with internal phloem and oil glands (Ciccarelli *et al.* 2008; Cronquist 1981; Metcalfe and Chalk 1979). Myrtaceae have polystemonous, mostly bisexual, actinomorphic flowers (Wilson 2011) with partially inferior to fully inferior ovaries (Conti *et al.* 1996) and usually with a nectariferous hypanthium (Johnson and Briggs 1984). The fruits are generally classified as either fleshy (berry) or dry (capsular or nut-like), but several other fruit types, such as drupes, and various intermediates, also occur in this family (Wilson

2011). The monophyletic tribe Myrteae (*sensu* Wilson *et al.* 2005; Biffin *et al.* 2010) comprises most of the fleshy-fruited Myrtaceae, including the ca. 2500 South American species. In ecological terms, Myrteae species generally occur in humid rainforests or flooded environments, usually wet gullies or streams (Kausel 1944, 1957).

The genus *Myrceugenia* Berg includes 40 species, 14 of which occur in Chile and the remaining in southeastern Brazil (Landrum 1981a; Murillo-A *et al.* 2012). The Chilean species of *Myrceugenia* are distributed from the semi-arid centre-northern region to the moist temperate forests in the southern tip of South America, as well as the Juan Fernandez Islands (Landrum 1981a, 1988). These species are an important component in the upper and middle strata of the temperate forests (Hildebrand-Vogel 2002).

Myrceugenia rufa (Colla) Skottsb. ex Kausel (Myrtaceae: Myrteae) is a shrub 1-2 m high, with reddish-brown hairs on leaves, peduncles, sepals and fruits. The leaves are small, thick, coriaceous, densely pubescent beneath and puberulent above. Peduncles are uniflorous, densely pubescent, solitary or 2-3 in a row in the axils of leaves. Flowers are bisexual, with suborbicular and pubescent calyx lobes and petals, and numerous stamens. The fruits are fleshy, yellowish to orange and pubescent (Landrum 1981a, 1988). The seeds are poorly known; they are frequently eaten by insects (Kausel 1944; Landrum 1981a, 1988).

This species is an exception in the fleshy-fruited Myrtaceae regarding its habitat preference. The species only occurs in the coast of central-north of Chile, in fragmented and open bushlands along ca. 200 km in the coastline and only few hundreds of meters inland (Landrum 1981a). In this dry habitat, the primary sources of moisture is oceanic breeze from the Pacific Ocean, and some occasional winter rain fall (Landrum 1981a; Serra *et al.* 1986). Due to this habitat, pubescence and growth habit, the species is considered adapted to xeric environments (Landrum 1981a). *Myrceugenia rufa* is considered "Rare" by the Red Book on Chilean Terrestrial Flora (Benoit 1989) and “near threatened by the current legislation (MMA, 2013), due to its scarcity and fragmented habitat (Kausel 1957). The main threats to the populations stem from urban development and fires (Hechenleitner 2005).

Although several anatomical studies have been performed in Myrtaceae, information regarding *Myrceugenia* is scarce and mainly limited to wood anatomy (Janssonius *et al.* 1908; Landrum 1981a; Metcalfe and Chalk 1979; Ragonese 1978; Record

and Hess 1943; Schmid and Baas 1984). Leaf anatomy has only been studied in three Brazilian species by Cardoso *et al.* (2009). The xerophytic condition of *M. rufa* has never been investigated anatomically. A leaf anatomical and micromorphological study of the species could enhance the understanding of the possible adaptations to drier environments in the family Myrtaceae.

This investigation has three aims: (1) Undertake a complete anatomical and micromorphological description of leaves of *M. rufa* for the first time, (2) determine if these descriptions match or differ from that of other fleshy-fruited South American Myrtaceae and (3) assess whether anatomical characters contradict or confirm the xerophytic strategy associated with this species.

MATERIAL AND METHODS

SPECIMEN SAMPLING

Sampling was conducted between November 2008 and February of 2014 aiming at representing the entire distribution range of the species. Fully developed leaves from forty-two specimens were collected from the following localities: Quebrada de Cordoba (El Tabo, V Region of Valparaíso; 33°20’S), Los Molles (V Region of Valparaíso; 32°40’S), Cerro de la Cruz (Zapallar, V Region of Valparaíso; 32°33’S), Rodelillo (Viña del Mar, V Region of Valparaíso; 33°00’S) and Cerro Talinay, IV Region of Coquimbo; 30°50’S). Samples from cultivated specimens were obtained from the National Botanical Garden (Viña del Mar). Vouchers were collected for representative specimens from certain localities. The material was identified according to Kausel (1944) and Landrum (1981a, 1988).

Specimens (Reta101, Reta102, Reta103 and Reta104) are currently deposited at the Queensland Herbarium (BRI), Brisbane, Australia. Duplicates (Reta101-2, Reta102-2, Reta103-2, Reta104-2) will be deposited at SGO (Museo Nacional de Historia Natural, Chile) and EIF (Facultad de Ciencias Forestales, Universidad de Chile) before publication.

LEAF ANATOMY

Preparation of histological samples for light microscopy (LM) followed Johansen (1940), Feder and O’Brien (1968), Ruzin (1999) and protocols previously used for Myrtaceae (Belsham and Orlovich 2003; Donato and Morretes 2009; Schmid and Baas 1984; Soh and

Parnell 2011). The samples were fixed in FAA for 48 h, dehydrated through ethanol series, followed by xylene-ethanol combinations (1:3, 1:2, 1:1, 1:0) 2 h each, infiltrated and embedded in paraffin wax. Sections of 5 µm thick were cut with an E. Leitz Wetzlar rotary microtome. Sections were stained with safranine O (1%), fast green, ferric chloride and tannic acid for various periods of time (Johansen 1940). The slides were sealed with Eukitt (acrylic resin-xylene) mounting medium.

LEAF MICROMORPHOLOGY

Fragments of leaves were fixed in FAA for 24-48h, dehydrated using a graded ethanol series and then critical point dried (Anderson 1951) in an Autosamdri-815 automatic critical point drier. Samples were then mounted on stubs with self-adhesive double-sided carbon discs and sputter-coated with gold palladium during 175 sec using a Leica EM SCD005 Gold Coater. Examination and photography were conducted using a FEI Quanta 200 SEM/ESEM operated at 10kV.

OBSERVATION AND MEASUREMENTS

Anatomical slides were examined with a Carl Zeiss Axiostar 10-031 microscope equipped with a Canon Power Shot A640 digital camera. Cells were measured and counted on digital micrographs with the UTHSCSA ImageTool 3.0 software (Wilcox *et al.* 2009). Cells and structures dimensions were calculated with 35 random repetitions in different samples of the 42 specimens, in order to obtain representative mean values. Measurements were calculated in micrometers (µm) and millimetres (mm) depending upon structure or cell type. Botanical terminology was based on Esau (1953) and Raven *et al.* (2005). Myrtaceae-specific anatomical descriptions and terminology were based on Schmid (1980), Schmid and Baas (1984), Cardoso *et al.* (2009) and da Silva *et al.* (2012)

RESULTS

MICROMORPHOLOGY OF LEAVES

Leaves of *M. rufa* are hypostomatic. Stomata are 8–21 µm long. Leaf stomatal density was determined as 632 ± 15 stomata per mm^2. The adaxial surface is rich in cuticular waxes, which are highly sinuous and give to the leaf a rough and rugged appearance. The adaxial epidermal cells are irregularly rounded and the anticlinal cell walls are strongly sinuous. On this surface, some scattered hairs are observed growing through wax (Fig. 1 A). Hairs are

125 ± 15.2 μm long. Hairs are unicellular, non-glandular, solitary, dibrachiate, appressed, conical, slightly wavy and persistent (Fig. 1 B). The midrib is strongly impressed on the adaxial surface and slightly prominent on the abaxial side. Secondary veins are not visible. The abaxial surface is pubescent, with a high density of dibrachiate, apressed and unicellular hairs (Fig. 1 C). The density of hairs covers the stomata almost completely. There are compacted groups of hairs apparently covering substomatal chambers on this surface (Fig. 1 D). The edges of the leaves are slightly revolute.

ANATOMY OF LEAVES

Myrceugenia rufa has dorsiventral leaves covered by a double epidermis of 26 ± 2.4 μm on the adaxial side (Fig. 2 A). A prominent cuticle of 6.6 ± 1.2 μm thick covers the adaxial surface of the leaf. On the abaxial side the cuticle is 3.6 ± 0.8 μm thick. Epidermal cells are compressed, plano-convex and mainly isodiametric. Stomata are anomocytic (*sensu* Gifford and Foster 1989), and large sub-stomatal chambers on the abaxial side are observed in some samples (Fig. 2 A), most times covered by hairs. Some stomata are partially hidden in crypts or depressions on the leaf surface. In transverse section, apressed unicellular hairs are visible on the abaxial surface (Fig. 2 A).

The mesophyll is formed by a compressed 2-4 layered palisade parenchyma and a spongy parenchyma with intercellular spaces. The palisade parenchyma layer is dense and composed of rectangular, attenuated and vertical cells. These cells possess thin primary cell walls and numerous chloroplasts. The spongy parenchyma is composed of irregular shaped cells (rounded to polygonal) (Fig. 2 B). Intercellular spaces correspond to 50-60% of the transverse area of the mesophyll. Secretory ducts are schizogenous and its average diameter is 95 ± 15 μm. These cavities are composed of large spaces surrounded by a sheath of peripheral epithelial cells, which are almost degenerated. Secretory ducts are abundant in all unlignified tissues and have variable dimensions (Fig. 2 C). Idioblasts with calcium oxalate crystals (druses) with a diameter of 25.1± 3.8 μm, occur throughout the palisade and spongy parenchyma (Fig. 2 D). An extension of the bundle sheath, composed of rounded-polygonal cells, is visible under the midrib. Internal phloem, as a continuous layer or isolated strands occurs on the adaxial side of vascular bundles (Fig. 2 E). Xylem vessels of the midrib show helicoidal thickenings of lignified secondary cell walls and scalariform

perforation plates. Phloem sieve tubes and companion cells have thin primary cell walls. Phloem fibres have evident and thick secondary cell walls.

DISCUSSION

Myrceugenia rufa shares a number of anatomical features with other Myrtaceae. These characters include druses (calcium oxalate crystals), internal phloem and secretory cells. Calcium oxalate crystals are abundant in the leaves of *M rufa,* especially in the palisade parenchyma, just below the adaxial epidermis. Druses are widely present in several genera of Myrtaceae, in diverse vegetative and reproductive structures. Donato and Morretes (2007) and Alves *et al.* (2008) described druses of calcium oxalate in South American species of *Eugenia.* Donato and Morretes (2011) reported the same structures for *Myrcia multiflora*. Polyhedral crystals, including druses, have been reported in *Psidium, Eugenia, Gomidesia* and *Myrcia*, among others (Cardoso *et al.* 2009; Gomes *et al.* 2009). The function of these structures is not completely clear, but has been related to the regulation of calcium and other minerals (Volk *et al.* 2002), as well as protection against herbivores and pathogens (Franceschi and Nakata 2005; Korth *et al.* 2006).

Internal phloem was found in all vascular bundles of leaves, either as continuous tissue or strands in the adaxial side of the midrib. This character is regarded as a typical anatomical character in the order Myrtales (Cronquist 1981; Takhtajan 1980) and is widely present in Myrtaceae (Schmid 1980; Cardoso *et al.* 2009).

It is relevant to know whether internal phloem is derived from the procambium, procambially derived or pith/mesophyll cells. This developmental difference may be important at the specific or generic level and it is regarded as a potential taxonomic character (Patil *et al.* 2009). The ontogeny of this tissue has not been studied in *Myrceugenia*.

Helical wall thickenings on vessel elements wall have been reported in a number of Myrtaceae genera, such as *Myrceugenia, Myrtus, Austromyrtus, Myrcia, Myrcianthes* and *Psidium* (Schmid and Baas, 1984). Similarly, scalariform perforation plates on the end wall of vessels in *Myrceugenia, Luma, Tepualia, Ugni, Neomyrtus* and *Myrtastrum* have been identified (Schmid and Baas 1984). Scalariform perforation plates and helical wall

thickenings on vessel walls, both found in *M. rufa*, have been attributed to putatively primitive species (Stern 1978). The latter, possibly as an adaptation of a common ancestor to cooler or mountain environments (Jansen *et al.* 2004). However, this has not been tested in detail in a phylogenetic context for the species.

The secretory ducts follow the typical schizogenous pattern commonly observed in Myrtaceae (Alves *et al.* 2008; Donato and Morretes 2007; Gomes *et al.* 2009). Has been reported that the ontogeny of secretory ducts in *Myrtus communis* follows an schizolysigenous development, which begins as lysigenous and ends as schizogenous (Cicarelli *et al*. 2008). Developmental studies should be conducted on *M. rufa* in order to confirm this double pattern. Volatile oils produced by secretory cavities in Myrtaceae have been identified as flavonoids (Wollenweber *et al.* 2000) and terpenoids (Judd *et al.* 1999; Lee 1998; Tanaka *et al.* 1996). Moreover, the occurrence of three types of secretory colleters (petaloid, conic and euryform) has been reported in different tribes of Myrtaceae, including Myrteae (da Silva *et al.* 2012). These structures were not observed on *M. rufa*.

Although the leaf anatomy of *M. rufa* shows similarities with that of other Myrtaceae species, the observed traits appear to be mainly associated with adaptations to an arid environment. Unlike almost all other species of Myrtaceae, the leaf epidermis is double-layered instead of being single-layered. The presence of a single epidermis has been reported for *Eugenia* (Armstrong *et al.* 2012; Donato and Morretes 2007; Donato and Morretes 2009; Esposito-Polesi *et al.* 2011; Fontenelle *et al.* 1994), *Myrcia*, *Campomanesia* (Gomes *et al.* 2009), *Callistemon, Eucalyptus, Melaleuca* (Tantawy 2004), *Acmena*, *Syzygium*, *Heteropyxis, Tristania* (Keating 1984) and the Chilean genus *Amomyrtus* (Retamales and Naulin 2010). A single epidermis is widely associated with mesophytic and hydrophytic species and it is considered the normal type of epidermis in vascular plants (Dickison 2000). *Myrceugenia euosma*, regarded as morphologically similar to *M. rufa*, possesses a hypodermis on the adaxial side (Cardoso *et al.* 2009). *Psidium* and *Pimenta* are other two genera of the family reported to possess a double epidermis (or possibly hypodermis) (Cardoso *et al.* 2009; Gomes *et al.* 2009; Tantawy 2004). There are no developmental or ontogenetic studies of leaf epidermis in Myrteae, in order to confirm whether the origin of this tissue is protodermal (double epidermis) or from the ground meristem (hypodermis). An unusually thick cuticle was observed in *M. rufa*, being close to 50% thicker than that observed in mesophytic Chilean Myrtaceae, such as *Amomyrtus luma*

and *A. meli* (Retamales and Naulin 2010). A thick cuticle and a multilayered epidermis are normally related to xerophytic plants, which prevent water losses due to excessive evapotranspiration (Dickison 2000; Esau 1953; Metcalfe and Chalk 1979). However, some studies suggest that the concentration of hydrophobic compounds in the cuticle are more important than the overall thickness of the cuticle for drought tolerance (Price 1982). In the case of Myrtaceae, this assumption has not been studied. Leaves of *M. rufa* possess scattered hairs on the adaxial surface and a dense layer of hairs on the abaxial side. The occurrence of a dense layer of hairs and other trichomes is regarded as a xerophytic adaptation (Esau 1953; Raven *et al.* 2005). Unlike *M. rufa*, leaves of Myrtaceae are often glabrous or possess scattered hairs on midribs and leaf blades (Wilson 2011). *M. eousma* is a species of the genus that has also a dense layer of hairs on the abaxial surface (Cardoso *et al.* 2009). In plants, the presence of hairs extends the boundary layer in a leaf (Ehleringer 1985) which creates a stable microclimate on the surface and reduces water losses due to excessive solar radiation (Riederer and Schreiber 2001). Large substomatal chambers covered by hairs are another feature commonly found in xerophytic species, but not usual in Myrteae. *M. eousma* also shows large substomatal chambers on the abaxial side, which is covered by hairs (Cardoso *et al*. 2009).

The presence of vertical and thin palisade cells in the mesophyll, as those found in this study, might be related to evolution of plants in open spaces, as bushlands (Chatelet *et al.* 2013).

CONCLUSION

In this study, the leaf anatomy and micromorphology of *Myrceugenia rufa* has been described for the first time. There are anatomical similarities between the species and other Myrtaceae taxa, particularly in terms of typical characters in the family. Additionally, this paper has explored the possible xeromorphic condition of the taxon based on character differences with the common anatomy of mesophytic Myrtaceae.

The anatomical study supports the xerophytic environmental adaptations of *M. rufa* including coriaceous leaves, a thick cuticle, double-layered epidermis, a dense palisade parenchyma, abundant hairs, and partially hidden stomata. These anatomical characters are

unusual in Myrtaceae, particularly in South American species. Normally, Myrtaceae taxa possess mesophytic or hydrophytic adaptations, opposite to the features described in this study. Some anatomical characters shared with other non-xerophytic Myrtaceae are oil secretory ducts, idioblasts containing druses and internal phloem in vascular bundles. Qualitative characters, particularly xerophytic, were consistent and did not show great variation across the individuals studied. Even samples from cultivated specimens did not show qualitative variation on xerophytic characters.

The origin of *M. rufa* might be related to geographic isolating events in southern South America 10-20 million years ago, such as the uplift of the Andes during the Miocene and the presence of the formation of the Arid Diagonal of South America (Landrum 1981a, 1981b; Hinojosa and Villagrán 1997). These conditions could explain the evolution of endemic taxa with characteristics differing from the common hydrophytic-mesophytic Myrtaceae. Whether the xerophytic characteristics of *M. rufa* are autapomorphies or shared traits with other *Myrceugenia* is unclear. Recent molecular phylogenetic analyses of *Myrceugenia* indicate that *M. rufa* is sister group to all the other Chilean and Brazilian species of the genus (Murillo-A et al, 2013).

Incorporating these morpho-anatomical findings in future studies, particularly phylogenetic analyses, can provide a better understanding of the evolution of this species and other anomalous taxa within the family. The comprehensive use of morpho-anatomical characters in a broad phylogeny of Myrtaceae including all the species of *Myrceugenia* is recommended. For this purpose, future anatomical studies will include *Myrcianthes coquimbensis* and all the species of Chilean *Myrceugenia*. This approach would allow testing whether the evolution of Myrtaceae species with xerophytic features is convergent or homologous and would also contribute to the evidence that can be used in evolutionary studies.

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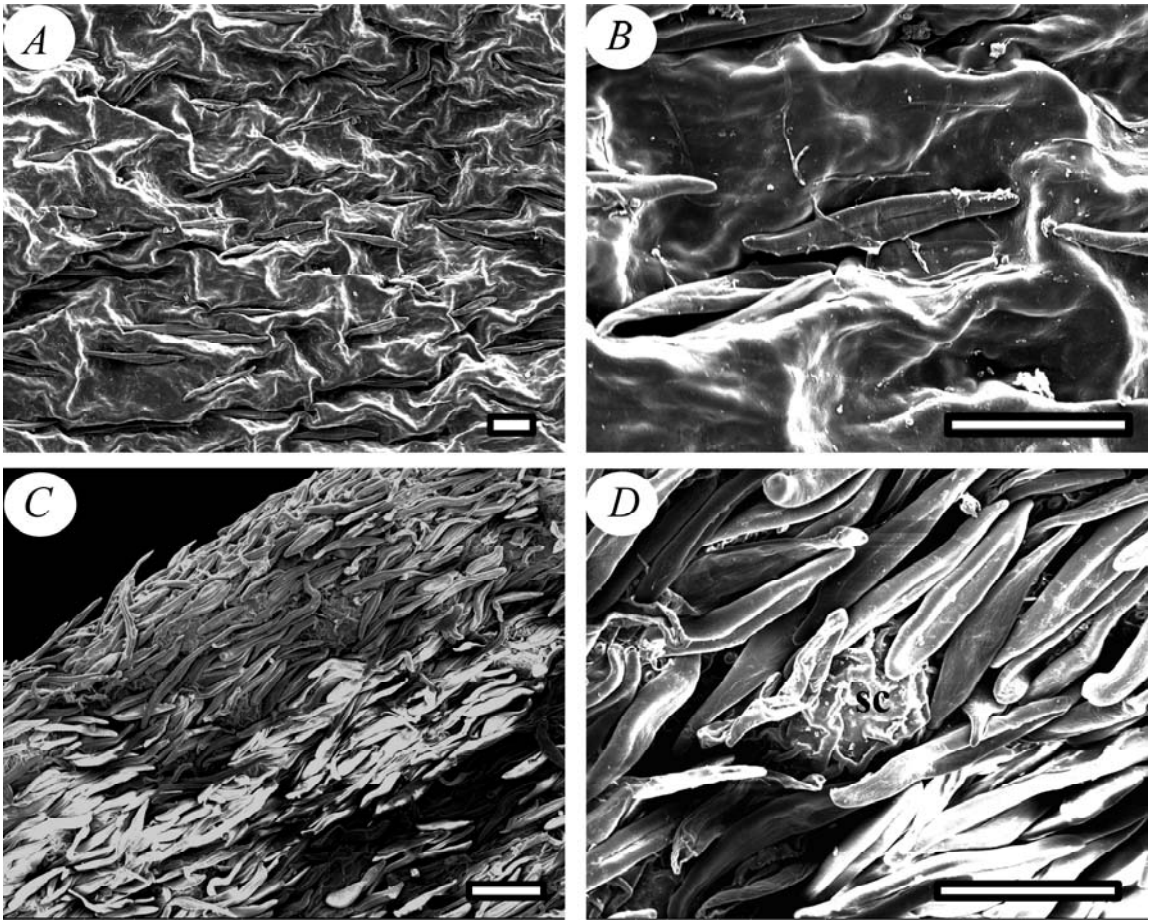

Fig 1. Microfotografías electrónicas de barrido (SEM) de las hojas de *M. rufa*. *A,* Superficie adaxial mostrando células epidermales, cera epicuticular y pelos dispersos. *B,* Detalle de los pelos en la superficie adaxial. *C,* Superficie abaxial con alta densidad de pelos unicelulares y no glandulares. *D,* Detalle de cavidad subestomática cubierta de pelos. **sc**- cavidad subestomática, Barra = 100 µm.

Fig. 1. Scanning electron micrographs of leaves of *M. rufa*. *A,* Adaxial surface showing epidermal cells, epicuticular wax and scattered hairs. *B,* Details of hair on the adaxial surface. *C,* Abaxial surface with high density of dibrachiate hairs. *D,* Detail of a substomatal chamber covered by hairs. **sc**- stomatal chamber, Scale bars = 100 µm.

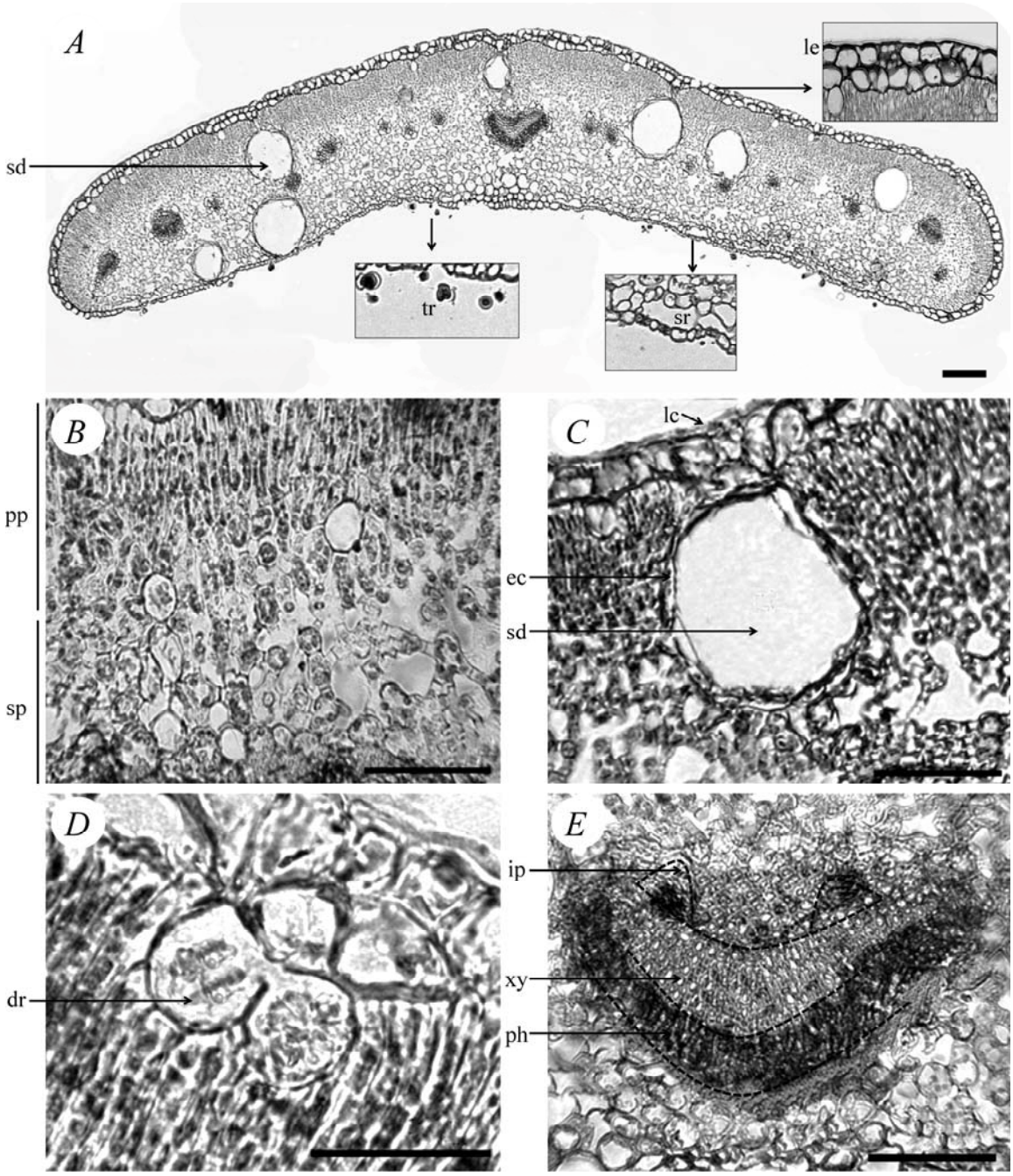
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Fig. 2. Light micrographs of transverse sections of the leaves of *M. rufa*. *A*, General view of the anatomy of the entire leaf blade; insets show multicellular hairs (tr), large substomatal chambers (sr) and multiple epidermis (le). *B*, Details of the mesophyll showing compressed palisade and spongy parenchyma. *C*, Detail of secretory duct and epithelial cells. *D*, Prominent idioblasts containing druses (calcium oxalate crystals) below the double epidermis and surrounded by the mesophyll. *E*, Section through the midrib showing the vascular bundle with internal phloem. Dotted lines in *E* show differentiation between xylem and phloem in the midrib. **dr**- druse, **ec**- epithelial cell, **lc**- leaf cuticle, **le**- leaf epidermis**, ip**- internal phloem, **ph**- phloem**, pp**- palisade parenchyma, **sd**- secretory duct, **sp**- spongy parenchyma, **sr**- substomatal chambers, **tr**- trichomes (hairs), **xy**- xylem. Scale bars = 50 µm.

Fig. 2. Microfotografías ópticas de secciones transversales de hoja de *M. rufa*. *A*, Vista general de la anatomía foliar; recuadros muestran pelos unicelulares (tr), cavidades subestomáticas amplias (sr) y epidermis múltiple (le). *B*, Detalle del mesófilo mostrando parénquima en empalizada y esponjoso. *C*, Detalle de cavidad secretora esquizógena con células epiteliales. *D*, Prominentes idioblastos con drusas (cristales de oxalato de calcio) bajo la epidermis y rodeados por el mesófilo. *E*, Sección transversal del haz vascular central con floema interno. Líneas punteadas en *E* indican diferenciación entre xilema y floema en el haz central. **dr**- drusa, **ec**- células epiteliales, **lc**- cutícula foliar, **le**- epidermis foliar**, ip**- floema interno, **ph**- floema**, pp**- parénquima en empalizada, **sd**- cavidades secretoras, **sp**- parénquima esponjoso, **sr**- cavidad subestomática, **tr**- tricomas (pelos), **xy**- xilema. Barra = 50 µm.